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Complete amino acid sequence of the large subunit of the low-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease (μCANP) deduced from its cDNA sequence

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The complete amino acid sequence of the large subunit (catalytic subunit) of human low-Ca^{2+} requiring-calcium-activated neutral protease (μCANP) was deduced from its cDNA base sequence. It is composed of 714 amino acid residues and its sequence is highly homologous to the chicken CANP sequence determined previously. Human μCANP, like chicken CANP, has a clear 4-domain structure, and their fundamental structures are essentially the same, although their Ca^{2+} sensitivities are significantly different. The role of each domain in the Ca^{2+} sensitivity and protease activity of CANP is discussed on the basis of sequence comparison.

Ca^{2+}-activated neutral protease *Calpain* *Thiol protease* *cDNA cloning* *Sequence homology* *Ca^{2+}*

1. INTRODUCTION

Ca^{2+}-activated neutral protease (CANP, calpain, EC 3.4.22.17) is an intracellular thiol protease that is ubiquitously found in tissues of higher animals [1–3]. Two types of CANP exist in mammals, μCANP and mCANP, which respectively require micromolar and millimolar Ca^{2+} for their activity [1–3]. Both CANPs are composed of two subunits, a large (catalytic) (M_r 80000) one and a small (regulatory) (M_r 28000) one [1–3]. As the small subunit is common to both CANPs [4,5], the Ca^{2+} requirement of CANP is determined apparently by the large subunit [6]. We have already shown that the large subunit of chicken CANP consists of four domains, two of which were identified as a thiol protease domain and a calmodulin-like Ca^{2+}-binding domain [7]. Comparison of the structures of μ and mCANPs is important for clarifying the molecular basis of their different Ca^{2+} sensitivities. However, since only one molecular species of CANP, with an intermediate Ca^{2+} sensitivity, has been found in chicken [7–9], we started to analyze the structures of μ and mCANPs from mammalian sources. Recently, cDNA clones corresponding to the C-terminal Ca^{2+}-binding domains of rabbit μ and mCANPs were isolated [10]. Here we report the complete amino acid sequence of the large subunit of human μCANP, which was deduced from the nucleotide sequence of its cDNA clone isolated using rabbit μCANP cDNA as a probe.

2. MATERIALS AND METHODS

Total RNA was prepared from human skeletal

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muscle and spleen [11], and then poly(A)$^+$ RNA enrichment was performed by oligo(dT)-cellulose chromatography [12]. Two cDNA libraries were constructed. One was prepared from human skeletal muscle poly(A)$^+$ RNA by the conventional method, as in [7]. The other library was constructed from human spleen poly(A)$^+$ RNA. Double-stranded cDNA was synthesized as in [13], and fractionated on low-gelling-temperature agarose gel after *Eco*RI linker ligation. cDNA longer than 2.3 kb was collected and ligated to the vector, λgt10 [14]. Screening of the library was performed as in [12]. DNA was sequenced according to [15].

3. RESULTS AND DISCUSSION

3.1. *Identification and DNA sequence of the cDNA clone for the large subunit of human μCANP*

The cDNA library from human skeletal muscle was screened with a *Pst*I cDNA fragment for the large subunit of rabbit μCANP [10]. One positive clone (pHM42) was isolated from about 45000 transformants and sequenced. Further screening of about 4×10^5 plaques from the human spleen cDNA λgt10 library with the pHM42 insert as a hybridization probe yielded 18 positive clones. The clone (λ31) containing the longest insert was subjected to nucleotide sequence analysis after subcloning into the pUC8 vector (resulting subclone; p31). The restriction map for the cDNA inserts in clones pHM42 and p31 is shown in fig.1,

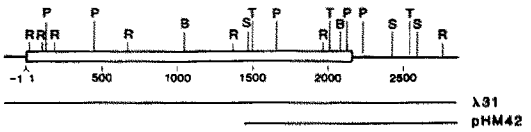

Fig.1. Restriction map of the inserts in cDNA clones p31 and pHM42 for the human μCANP large subunit. Horizonal lines denote regions covered by the inserts of p31 and pHM42. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG triplet that encodes the initiation methionine. An open box indicates the protein-coding region and horizontal lines on both sides of the box indicate the 5′- and 3′-untranslated regions. Vertical lines and letters represent restriction sites. R, *Rsa*I; P, *Pvu*II; B, *Bgl*II; S, *Sma*I; T, *Taq*I.

the total nucleotide sequence of cDNA (3011 bp) being shown in fig.2.

The translational initiation site was assigned to ATG at position 1, because a termination codon, TGA, exists at −57 in phase and the second ATG triplet appears a long way downstream (at position 778). This open reading frame is terminated by TGA at position 2143 and encodes 714 amino acid residues. The calculated M_r (81889) and the amino acid composition agree well with the reported values [16].

The C-terminal amino acid sequence of the large subunit of rabbit μCANP [10] is almost identical (97% homology) to the deduced sequence for the corresponding position. Furthermore, we have already obtained another cDNA clone that encodes the large subunit of human mCANP (unpublished). Thus, it is concluded that the present cDNA clones encode the large subunit of human μCANP. Northern blot hybridization of human spleen RNA with the cDNA insert of p31 as a probe gave a single band at a position corresponding to approx. 3.5 kb (fig.3). This indicates that the cloned cDNA, spanning a total of 3011 bp, covers nearly the full length of the μCANP mRNA.

3.2. *Comparison of the amino acid sequences*

This is the first complete sequence determination of the large subunit of μCANP. As for the structure of the large subunit of CANP, the complete sequence of chicken CANP [7] and the C-terminal sequences of rabbit m and μCANPs [10] have been elucidated.

Comparison of the deduced amino acid sequence of human μCANP with that of chicken CANP indicated that they are highly homologous, irrespective of the clear difference in their Ca^{2+} sensitivities, and human μCANP, like chicken CANP, has a clear 4-domain structure (I–IV from the N-terminus). In domain II, the protease domain (amino acid residues 88–327), two active-site amino acid residues, Cys-115 and His-272, are found, and the sequences around these residues are highly conserved among various cysteine proteinases [7,17,18]. In domain IV, the Ca^{2+}-binding domain (residues 570–714), 4 consecutive EF-hand sequences are found. The scores for the test sequence [19] are above 11 for all the 4 EF-hand sequences, indicating that these 4 sites can be regarded as potential Ca^{2+}-binding sites. The total

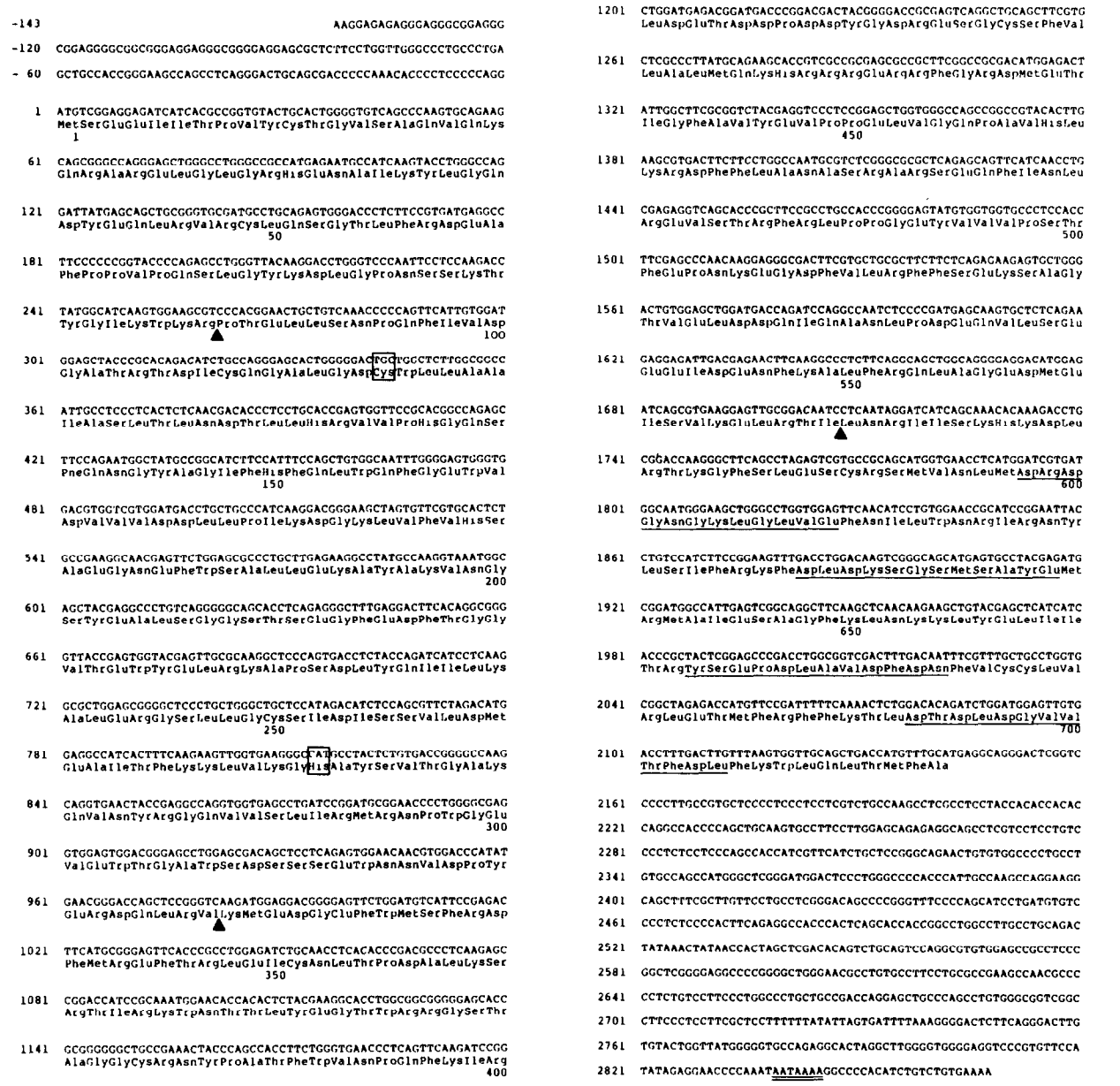

Fig.2. Nucleotide sequence of the large subunit of human μCANP cDNA and the deduced amino acid sequence. Two residues at the active site (Cys-115, His-272) are boxed. The amino acid residues corresponding to the Ca^{2+}-binding loop of the 4 EF-hand structures are underlined. Closed triangles indicate the boundaries of domains. The polyadenylation signal is double-underlined.

sequence homology between chicken CANP and human μCANP is 70%. Furthermore, the homologies of the two proteins are 54, 78, 72 and 65% for domains I, II, III and IV, respectively. The homology in domain II is higher than those for the other domains.

A clear structural difference was found between the N-terminal sequences of human μCANP and

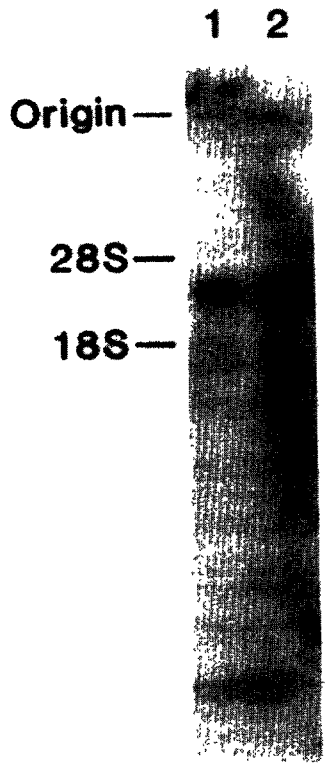

Fig.3. Blot hybridization of human spleen RNA. Poly(A)$^+$ RNA (lane 1, ~0.3 μg) and total RNA (lane 2, ~2.0 μg) were analyzed by the procedure described in [12] using nylon membrane. The probe used for hybridization was the *Rsa*I fragment (nos 1967–2763 in fig.2) labelled by nick-translation with [α-^{32}P]dCTP. The size marker was human rRNA.

chicken CANP. The former is larger than the latter by 7 amino acid residues. The Ca^{2+} sensitivity of chicken CANP increases significantly upon removal of the N-terminal 17-residue peptide by autolysis [7,18]. Thus, the N-terminal region is also important for the Ca^{2+} sensitivity of CANP. In this respect, a clear structural difference in the N-terminal region is noticeable.

From these results we assume that various CANPs have a common fundamental mechanism for the expression of protease activity and that their Ca^{2+} sensitivities are determined mainly by differences in the structure of the C-terminal Ca^{2+}-binding domain (domain IV). The N-terminal region (domain I) of native CANP might interact with domains II and/or IV, and thus repress the Ca^{2+}-dependent proteolytic activity. Autocatalytic cleavage of the N-terminal region affects the interaction between functional domains and changes the Ca^{2+} sensitivity of CANP.

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REFERENCES

[1] Imahori, K. (1982) in: Calcium and Cell Function (Cheung, W.Y. ed.) vol.3, pp.473–485, Academic Press, New York.
[2] Murachi, T. (1983) in: Calcium and Cell Function (Cheung, W.Y. ed.) vol.4, pp.377–410, Academic Press, New York.
[3] Suzuki, K., Kawashima, S. and Imahori, K. (1984) in: Calcium Regulation in Biological Systems (Ebashi, S. et al. eds) pp.213–226, Academic Press, New York.
[4] Yumoto, N., Kikuchi, T., Sasaki, T. and Murachi, T. (1984) J. Biochem. 96, 1531–1537.
[5] Kawasaki, H., Imajoh, S., Kawashima, S., Hayashi, H. and Suzuki, K. (1986) J. Biochem. 99, 1525–1532.
[6] Kikuchi, T., Yumoto, N., Sasaki, T. and Murachi, T. (1984) Arch. Biochem. Biophys. 243, 639–645.
[7] Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984) Nature 312, 566–570.
[8] Kawashima, S., Nomoto, M., Hayashi, M., Inomata, M., Nakamura, M. and Imahori, K. (1984) J. Biochem. 95, 95–101.
[9] Suzuki, K., Ohno, S., Imajoh, S., Emori, Y. and Kawasaki, H. (1985) Biomed. Res. 6, 323–327.
[10] Emori, Y., Kawasaki, H., Sugihara, H., Imajoh, S., Kawashima, S. and Suzuki, K. (1986) J. Biol. Chem. 261, 9465–9471.
[11] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[12] Maniatis, T., Fritsch, E.F. and Sambrook, J.

(1982) in: Molecular Cloning, Cold Spring Harbor Laboratory, NY.
[13] Gubler, U. and Hoffman, B.J. (1983) Gene 25, 263–269.
[14] Huynh, T., Young, R. and Davis, R.W. (1985) in: DNA Cloning, A Practical Approach (Glover, D. ed.) vol.1, pp.49–78, IRL Press, London.
[15] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[16] Hatanaka, M., Kikuchi, T. and Murachi, T. (1983) Biomed. Res. 4, 381–388.
[17] Suzuki, K., Hayashi, H., Hayashi, T. and Iwai, K. (1983) FEBS Lett. 152, 67–70.
[18] Suzuki, K., Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H. and Kisaragi, M. (1985) in: The Biological Role of Proteinases and Their Inhibitors in Skin (Ogawa, H. et al. eds) pp.111–120, University of Tokyo Press, Tokyo.
[19] Tufty, R.M. and Kretsinger, R.H. (1975) Science 187, 167–169.